

Autoreactive T cells specific for insulin B:11-23 recognize a low-affinity peptide register in human subjects with autoimmune diabetes

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Previous studies in type 1 diabetes (T1D) in the nonobese diabetic mouse demonstrated that a crucial insulin epitope (B:9-23) is presented to diabetogenic CD4⁺ T cells by IA^{g7} in a weakly bound register. The importance of antigenic peptides with low-affinity HLA binding in human autoimmune disease remains less clear. The objective of this study was to investigate T-cell responses to a low-affinity self-epitope in subjects with T1D. HLA-DQ8 tetramers loaded with a modified insulin peptide designed to improve binding the low-affinity register were used to visualize T-cell responses following in vitro stimulation. Positive responses were only detectable in T1D patients. Because the immunogenic register of B:9-23 presented by DQ8 has not been conclusively demonstrated, T-cell assays using substituted peptides and DQ8 constructs engineered to express and present B:9-23 in fixed binding registers were used to determine the immunogenic register of this peptide. Tetramer-positive T-cell clones isolated from T1D subjects that responded to stimulation by B:11-23 peptide and denatured insulin protein were conclusively shown to recognize B:11-23 bound to HLA-DQ8 in the low-affinity register 3. These T cells also responded to homologous peptides derived from microbial antigens, suggesting that their initial priming could occur via molecular mimicry. These results are in accord with prior observations from the nonobese diabetic mouse model, suggesting a mechanism shared by mouse and man through which T cells that recognize a weakly bound peptide can circumvent tolerance mechanisms and play a role in the initiation of autoimmune diseases, such as T1D.

antigen presentation | self-antigen | MHCII tetramers

Type 1 diabetes (T1D) is a polygenic T-cell-mediated autoimmune disease with strong genetic linkages to the MHC class II and insulin promoter regions (1). Class II molecules are fundamental for CD4⁺ T-cell activation, whereas the insulin promoter polymorphism can modulate insulin levels in the thymus thereby influencing the threshold of selection for insulin-specific autoreactive T cells (2, 3). In the nonobese diabetic (NOD) mouse model of autoimmune mediated diabetes, insulin-specific IA^{g7}-restricted CD4⁺ T cells have been strongly implicated in β -cell destruction. In prediabetic NOD mice, 50% of the T-cell clones established from islet-infiltrating lymphocytes were insulin-specific and the majority of these clones recognized the insulin B:9-23 (B:9-23 SHLVEALYLVCGERG) epitope (4, 5). Moreover, substitution of a single residue in the B:9-23 region abrogated development of diabetes in a transgenic mouse model (6, 7). Thus, in the murine NOD model, the IA^{g7}-restricted B:9-23 epitope is considered to be pivotal in the development of diabetes.

The position or “register” that B:9-23 occupies within the IA^{g7} binding groove has been a controversial but important question. At least three binding registers (R) have been considered, defined here by which B:9-23 amino acid occupies the first binding pocket (p1) position in the IA^{g7} binding groove: R1, p1 V12; R2, p1 E13; and R3, p1 A14. Each binding register creates a unique set of upward pointing amino acid side-chains for T-cell recognition

and also exhibits a distinct binding affinity for MHC class II conferred by the downward-facing amino acids that occupy its binding pockets. The Unanue group first reported that B:9-23 can be presented by IA^{g7} in either R1 or R2 and that each register is recognized by a different set of B:9-23-specific CD4⁺ T cells (8). In contrast, a separate study (9, 10) reported that T cells recognize the B:9-23 peptide bound to IA^{g7} in R3, the weakest binding of the three registers. Mutation of R22 to the highly p9 favorable E greatly improved the binding of the peptide to IA^{g7} and its presentation to CD4⁺ T cells. Disulfides introduced between the peptide and IA^{g7} confirmed the R3 binding. These data support the relevance of antigenic peptides with low-affinity HLA binding in the NOD model of diabetes.

In humans, the *HLA-DQB1*0302* allele confers high disease risk. The DQ8 molecule, which is comprised of the DQA1*0301 and DQB1*0302 chains, is the human homolog for IA^{g7}. In particular, both IA^{g7} and DQ8 have a basic pocket 9 (p9, because of having a non-Asp β 57 residue in the class II β -chain) that accommodates and is stabilized by peptides with acidic residues at p9 (11). Insulin autoreactive antibodies precede the onset of disease and have been used for risk stratification in susceptible individuals (12, 13). CD8⁺ T cells that are specific for insulin have also been detected (14, 15), and it has been documented that they are capable of lysing islets (16, 17) and there is one report of human CD4⁺ T cells responsive to an insulin A-chain peptide presented by

Significance

The importance of antigenic peptides with low-affinity HLA binding in human autoimmune disease remains unclear. Studies in the nonobese diabetic mouse demonstrate recognition of a crucial insulin epitope presented in a weakly bound register. This work details a direct study of responses to this insulin B-chain peptide in humans. Responses were readily detected in subjects with type 1 diabetes. Furthermore, T-cell clones were shown to recognize the peptide presented in a weakly bound alternative register. These findings confirm the relevance of immune recognition of this segment of the insulin B-chain in human disease and highlight a mechanism shared by mouse and man through which T cells that recognize a weakly bound peptide can circumvent tolerance mechanisms.

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HLA-DR4 (18). These data all suggest that insulin is also an important autoantigen in human T1D.

In the light of the accumulated data from both murine and human studies, it is logical to postulate that DQ8-restricted B:9-23-specific CD4⁺ T cells are present in human subjects with T1D and that this peptide may also be recognized in a weakly bound register. Although recognition of B:9-23 has long been suspected, direct evidence for the presence and importance of DQ8-restricted B:9-23-specific CD4⁺ T cells in human subjects with T1D is limited and no study has definitively established the immunogenic register of B:9-23 as presented by DQ8. Alleva et al. showed that T cells from the peripheral blood of DR4-DQ8 T1D subjects had measurable proliferative and IFN responses to the B:9-23 peptide (19). In addition, the proliferation of a B:9-23-specific T-cell line could be blocked through addition of an anti-DQ Ab (19). Eerligh et al. also reported the isolation of a DQ8 restricted Ins B:6-22 T-cell clone from a T1D subject (20). However, more detailed analysis of these responses, for example by direct visualization and cloning of multiple DQ8/B:9-23-specific cells, has remained a desirable but elusive goal at least in part because of the technical challenge of interrogating DQ8-restricted autoreactive T cells.

In the current study, our objective was to extend these previous observations by visualizing DQ8-restricted T-cell responses to B:9-23 in human subjects with T1D and determining the immunogenic register of the DQ8/B:9-23 complex. In particular, we wished to investigate responses to B:9-23 as a possible example of T-cell responses to a self-epitope with low-affinity MHC binding. We used DQ8/insulin tetramers to detect B:11-23-specific T cells in peripheral blood samples from T1D subjects and compared the frequency of these responses in T1D subjects and healthy controls with *DQ8* haplotypes. After directly cloning these T cells, we investigated their responsiveness to stimulation with insulin peptides, denatured insulin protein, and homologous peptides derived from bacterial antigens. By assessing proliferation in response to peptides with alanine or phenylalanine substitutions, we identified a clear immunogenic register within B:11-23 that is bound by DQ8 with low affinity and recognized by human T cells.

Results

DQ8-Restricted Responses to B:11-23 Are Detectable in Subjects with T1D but Not Healthy Controls. We first used an in vitro tetramer assay to directly visualize DQ8-restricted T-cell responses to insulin B:11-23 in subjects with T1D and controls. A B:9-23 peptide with a R22E substitution (B:9-23^{R22E}) was previously shown to be more potent in activating a set of B:9-23 hybridomas from NOD mice compared with the wild-type B:9-23 peptide (9, 10). This modification enhances binding to IA^{g7} in the R3 register by about 50-fold (9). Because the human and mouse B:11-23 amino acid sequences are identical, we used an HLA-DQ8 tetramer loaded with the B:11-23^{R22E} peptide to enhance expansion and detection of responsive T cells. Samples from 16 T1D patients, 10 healthy controls, and 2 control subjects with type 2 diabetes (who needed daily insulin injections)—all with *DQ8* haplotypes—were evaluated for insulin responses. Peripheral blood mononuclear cell from these subjects were stimulated with either B:11-23^{R22E} or B:11-23 peptide. The cells were cultured for 2 wk and assayed for the presence of B:11-23-specific T cells using DQ8/B:11-23 and DQ8/B:11-23^{R22E} tetramers. Specific responses as assayed by DQ8/B:11-23^{R22E} tetramers were significantly more prevalent in subjects with diabetes than in healthy controls, with detectable responses in 6 of 16 T1D subjects (Fig. 1) and 0 of 12 *HLA-DQ8*-matched non-T1D controls (Fisher's exact test, $P = 0.0237$). Among the positive responses, five were from B:11-23^{R22E} stimulation and one from B:11-23 stimulation. Staining results for DQ8/B:11-23 tetramer were negative in this set of experiments, suggesting that the R22E

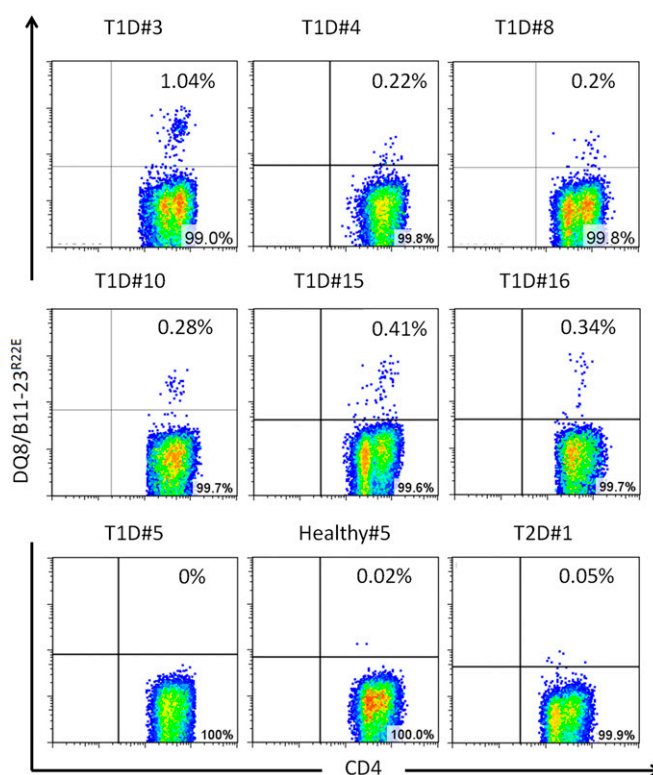


Fig. 1. Detection of insulin-specific T cells following in vitro expansion. CD4⁺ T cells from T1D patients, type 2 diabetes (T2D) patients, and healthy control subjects with *DQ8* haplotypes were individually stimulated with B:11-23 or modified B:11-23^{R22E} for 14 d. Antigen-specific T cells were then detected by staining with HLA-DQ8/B:11-23^{R22E} tetramer. Each panel depicts a representative subject (patient ID listed above each panel) which had a positive tetramer staining result. Cells from subject T1D#10 were stimulated with B:11-23 peptide and the rest were stimulated with B:11-23^{R22E}. Subjects T1D#5, Healthy#5, and T2D#1 provide examples of negative tetramer staining.

substitution is necessary to produce an effective tetramer. Responses in DQ8⁺ control subjects with type 2 diabetes were negative, suggesting that injection with exogenous insulin was not sufficient to elicit a response to B:11-23^{R22E} stimulation. Interestingly, five of the six subjects who responded to B:11-23 had the susceptible *VNTR I/I* genotype that has been associated with reduced insulin levels in the thymus (2) (Table S1).

Characterization of DQ8-Restricted Insulin-Reactive CD4 T Cells. A total of 26 T-cell clones were obtained from five different subjects with T1D by single-cell sorting and expanding of DQ8/B:11-23^{R22E} tetramer-positive cells. Eight of these clones were used for subsequent experiments. Fig. 2 shows representative results for one clone (results for additional clones were all similar). T-cell clones stained brightly with tetramer (Fig. 2A) and proliferated vigorously in response to B:11-23^{R22E} peptide (Fig. 2B and C). That proliferation was inhibited by anti-DQ blocking antibody (SPVL3), indicating an HLA-DQ-restricted response (Fig. 2B). Functional analysis of the clones by intracellular cytokine staining indicated that IFN- γ , IL-2, and TNF- α were the predominant cytokines produced (Fig. S1 and Table S2). Several clones also produced lesser amounts of IL-13 and IL-4, a few clones also had some IL-10, and one clone was positive for IL-5. The clones responded to stimulation with B:11-23 peptide and denatured insulin (deINS) protein (Fig. 2C). deINS delivered a stronger stimulatory activity at 1.7 μ M than at 17 μ M, probably because protein aggregates were inhibitory or toxic at 17- μ M concentration (Fig. 2C). The clones failed to proliferate in response to native

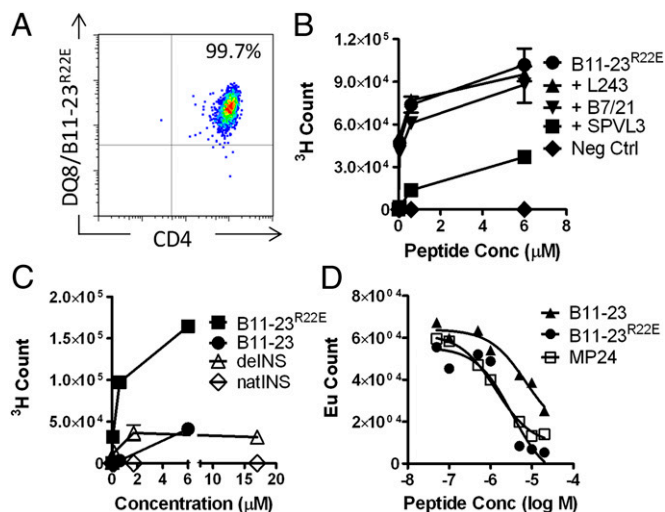


Fig. 2. HLA-DQ8-restricted T-cell clones isolated using B:11-23^{R22E} tetramers respond to wild-type insulin peptide and denatured insulin. (A) DQ8/B:11-23^{R22E} tetramer staining of a representative T-cell clone isolated from subject T1D#3. (B) ³H-Thymidine incorporation of T-cell clone T1D#3 C8 following stimulation with B:11-23^{R22E} peptide or irrelevant control peptide stimulation in the absence or presence of 5 μg/mL of L243 (HLA-DR blocking antibody), B7/21 (HLA-DP blocking antibody), or SPVL3 (HLA-DQ blocking antibody). Data are mean ± SD of duplicates. (C) Stimulation of a representative T-cell clone T1D#3 C8 by B:11-23^{R22E}, B:11-23, denatured or native insulin. Data are mean ± SD of duplicates. (D) In vitro binding of B:11-23, B:11-23^{R22E}, and influenza matrix protein (MP) peptides to DQ8. Competitive binding assays were carried out using a biotinylated reference GAD65 peptide and increasing concentrations of nonbiotinylated B:11-23, B:11-23^{R22E}, or MP24 (MP₁₈₅₋₂₀₄ TAKAMEQMAGSSEQAEEAME). Based on the binding curves, the IC₅₀ values were 10.4 μM for B:11-23, 1.6 μM for B:11-23^{R22E}, and 1.4 μM for MP24, respectively.

insulin protein (Fig. 2C). The responsiveness of eight different clones from four different subjects to B:11-23^{R22E} peptide, B:11-23 peptide, and deINS protein are summarized in Table 1. These data demonstrate that T cells isolated with DQ8/B:11-23^{R22E} tetramer are also specific for wild-type insulin B:11-23 peptide and respond to deINS. The stimulation index for clones stimulated with B:11-23 was approximately sevenfold lower than clones stimulated with an equal concentration of B:11-23^{R22E} peptide. This finding is in agreement with our observation that B:11-23^{R22E} (IC₅₀ = 1.6 nM) has a sevenfold higher affinity than B:11-23 (IC₅₀ = 10.4 nM) to the HLA-DQ8 molecule as measured using an in vitro peptide binding assay (Fig. 2D). Interestingly, when antigen-presenting cells (APCs) were primed with deINS protein (~1.7 μM) or B:11-23 (6 μM), deINS protein elicited a similar stimulation index compared with B:11-23 peptide (Table 1). This result indicates that an epitope that corresponds to the B:11-23 peptide can be processed from deINS protein and presented effectively on HLA-DQ8 to activate insulin-specific T cells.

Determining the Immunogenic Register of Insulin B:11-23 Bound to HLA-DQ8. To define the register of the B:11-23 peptide that is recognized as an immunogenic epitope by human T cells, two libraries of alanine substituted peptides with either B:11-23^{R22E} or B:11-23 as the prototypic peptide template were synthesized and the relative capacities of the substituted peptides to stimulate T-cell clones were compared. Fig. 3A shows the stimulation indexes of each peptide in the library using B:11-23^{R22E} as prototype for three clones (clone T1D#10 C8 was generated from wild-type B:11-23 stimulation, and clone T1D#3 C8 and T1D#4 C5 generated from B:11-23^{R22E} stimulation). Overall, this alanine substituted library induced a similar response pattern for all three T-cell clones examined. The major influence of alanine

substitution on stimulatory activities was present between residues A15 to E22. This finding suggests the region of the peptide that is recognized by these T cells is the A15 to E22 region. Further analysis revealed that substitutions of A14F, L17A, C19A, and G20A had minimal effect on stimulatory activity for T1D#10 C8 and T1D#4 C5 clones. Although substitutions of Y16A and E21A markedly reduced the stimulatory activity for all three clones, substitutions of L15A and V18A reduced the stimulatory activity of two of three clones. Based on this pattern of peptide stimulatory activity, it is likely that B:11-23^{R22E} bound to DQ8 using R3. In this scenario, residues A14, L17, and C19 correspond to the p1, p4, and p6 anchoring residues, respectively. The side chains of these anchor residues are buried within the HLA binding pockets and substitution of these residues would have limited effects on T-cell receptor (TCR) recognition. In contrast, residues L15, Y16, V18, and E21 would correspond to positions 2, 3, 5, and 8 of the HLA binding register, which are potential TCR contact residues; alanine substitutions at TCR contact positions would be expected to affect T-cell recognition, as was observed.

Clone T1D#4 C5 also showed increased responses to the E13A substituted peptide compared with wild-type peptide. E is a naturally preferred p1 anchor for DQ8, as described by Suri et al. (11). Therefore, replacement of E13 to A would eliminate the preferred p1 anchor of R2, increasing the likelihood that the peptide would anchor to other DQ8 registers such as in R3, thereby enhancing stimulatory activity. Clone T1D#3 C8 showed a slightly different reactivity profile compared with the other two clones. For this clone, L17A substitution abrogated activation, whereas V18A substitution had minimal effect. This finding could be interpreted to suggest that R2 is the immunogenic register recognized by this clone. In this scenario, L15, Y16, L17, and E22 would be potential T-cell contact residues (Fig. 3A). However, it is more likely that this clone still recognizes B:11-23^{R22E} presented in R3, because the overall pattern of stimulatory activity was very similar to the other two clones. This conclusion was further supported by the next set of data.

When T-cell clones were stimulated with the library using B:11-23 as the prototypic peptide, the stimulation activities were enhanced for all clones with peptides having substitutions of A14F and R22A. These data suggest that A14 is the p1 anchor residue, because F has been shown to serve as a better p1 anchor than A (21). Correspondingly, A22 would be the p9 residue, where A would be better accommodated than a basic R. These data do not support E13 as the p1 anchor for the recognition by these T-cell clones, because if E13 were anchored in p1, A14 would be at position 2 of the binding register and would likely influence TCR recognition. However, the results show that an A14F substitution did not decrease stimulatory activity for any of the T-cell clones. Instead this substitution enhanced the stimulatory activity for all

Table 1. Stimulation index of insulin specific T-cell clones in response to peptides and denatured protein

Clone no.	B:11-23 ^{R22E}	B:11-23	deINS
T1D#3 C5	485	122	190
T1D#3 C8	327	5.3	110
T1D#4 C5	189.6	77.7	54.2
T1D#4 C6	212.0	37.1	NT
T1D#10 C1	278.2	9.6	48
T1D#10 C3	302.2	10.2	46.5
T1D#10 C8	634.5	83	149
T1D#15 C2	346.9	31.4	NT
Average ± SD	347 ± 147	47 ± 43	100 ± 60

Reported values are stimulation index in response to stimulation with B:11-23^{R22E} peptide (6 μM), B:11-23 peptide (6 μM), or deINS protein (1.7 μM) versus an unstimulated control.

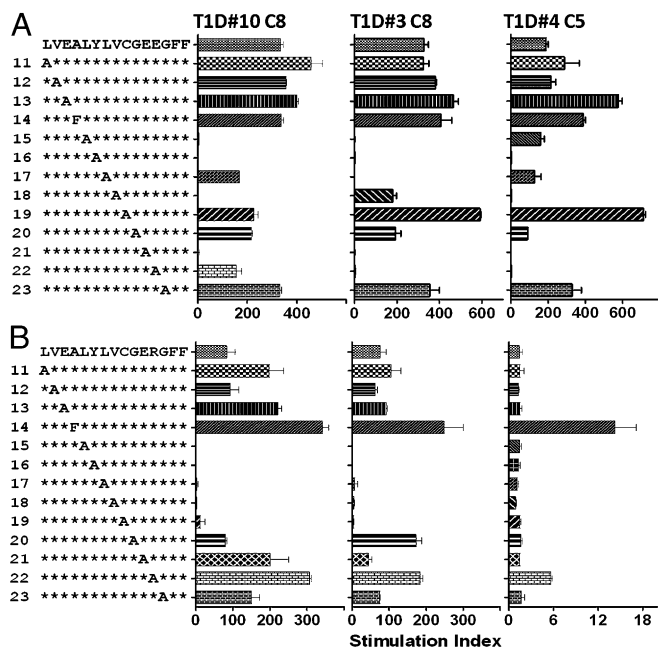


Fig. 3. Determining the immunogenic register of B:11-23 that is recognized by DQ8-restricted T cells. (A) Stimulation index for T-cell clones activated in vitro using B:11-23^{R22E} or its substituted derivatives (all clones were stimulated with 3- μ M peptides). The B:11-23^{R22E} peptide sequence is listed on the top of the y axis, followed by substituted peptides, with identical residues marked using the asterisk symbol. Alanine was used to replace each residue of the peptide with the exception of A14 where the wild-type alanine was replaced with F. Clone T1D#10 C8 was generated from wild-type B:11-23 stimulated cultures, and clone T1D#3 C8 and T1D#4 C5 were generated from B:11-23^{R22E}-stimulated cultures. Data are mean \pm SD of duplicates. (B) Stimulation index for T-cell clones activated in vitro using B:11-23 or its substituted derivatives (all clones were stimulated with 30- μ M peptides). The B:11-23 peptide sequence is listed on the top of the y axis, followed by substituted peptides, with identical residues marked using the asterisk. Alanine was used to replace each residue of the peptide with the exception of A14 where the wild-type alanine was replaced with F. Data are mean \pm SD of duplicates.

clones. Substituting R22 to A also enhanced stimulatory activity for all clones that could also potentiate binding in R3, because R is a relatively poor p9 anchor (compared with an A residue at p9) for DQ8 binding.

To further confirm that T-cell recognition of B:11-23 is specific to R3, T1D#10 C8, T1D#3 C8, and T1D#4 C5 T-cell clones were stimulated with DQ8 constructs engineered to express the insulin peptide covalently linked to the N terminus of DQB1*0302. The binding register of the peptide was fixed through formation of a disulfide bond between the DQ8 α -chain and the peptide (9). Two sets of such trapped constructs were generated, one with cysteine introduced at position 65 of the α -chain (V65C), designed to form a bond with a cysteine present at position p6 of the peptide, and another with an I72C mutation designed to bind to a cysteine at p11 (Fig. S2A). The peptides in different registers were also modified in such a fashion that both p1 and p9 would be an E; and any C that would not be at the p6 position was changed to an A. B6K10 cells were transduced with retroviral versions of these constructs for subsequent use as artificial APCs (22). The surface expression levels of DQ8/Ins complexes for both I72C and V65C series are similar except for the R4 variants of V65C, which showed a reduced surface expression (Fig. S2B). Western blot analysis of surface immunoprecipitates from the I72C series separated by SDS/PAGE under reducing or nonreducing conditions demonstrated correct formation of the peptide:DQ8 disulfide bond, thus confirming

trapping of the insulin peptide in the defined register (R1, R2, R3, or R4) (Fig. S2C). Consistent with our previous results, the three representative T-cell clones tested were only responsive to B6K10 lines expressing DQ8/Ins complexes fixed in R3 (Table 2). Overall, the results of these experiments clearly demonstrate that B:11-23-specific human DQ8-restricted T-cell clones that we isolated respond to the peptide bound in the R3 register, and not in R1, R2, or R4.

Insulin-Reactive T-Cell Clones Respond to the Microbially Derived Peptides. Among the possible mechanisms that could initiate autoimmune diabetes, it has been postulated that adaptive responses that are primed by microbial antigens could cross-recognize homologous self-epitopes. To investigate whether B:11-23-specific T cells can be activated by such homologous peptides, we performed a sequence homology search for ALYLVCGER among all bacteria, viruses, and fungi deposited within the National Center for Biotechnology Information protein database. We identified four microbial peptides with high-sequence homology to ALYLVCGER (Table S3). These sequences were derived from *Bacteroides salanitronis*, a common inhabitant of chicken intestine/cecum (23), *Clostridium acetobutylicum*, a soil dwelling *Clostridium* species (24), *Treponema phagedenis*, a pathogen of papillomatous digital dermatitis isolated from Iowa dairy cattle (25), and *Microscilla marina*, a bacterial group frequently found on marine particulates (26). All of the T-cell clones tested responded to stimulation with these bacterial peptides and the responses, as evaluated by the stimulation index, were of comparable magnitude to the modified B:11-23^{R22E} peptide (Fig. 4). These data imply that T cells that recognize B:11-23 on HLA-DQ8 could be effectively primed by microbial antigens.

Discussion

This study provides direct evidence that B:11-23 reactive CD4⁺ T cells are present in the peripheral blood of subjects with T1D and that the immunogenic register of this peptide has low-affinity binding to DQ8. Responses to B:11-23 by DQ8-restricted T cells were more readily detected in T1D subjects compared with healthy controls, visualizing these cells using DQ8/B:11-23^{R22E} tetramers following in vitro expansion. We further verified the specificity and relevance of these T cells by isolating tetramer-positive clones and documenting their responsiveness to the wild-type peptide and denatured insulin protein. B:11-23 reactive cells appeared to be present at relatively low frequencies in the subjects studied, as responses were modest (less than 1% of expanded CD4⁺ T cells and

Table 2. T-cell clones respond to register-fixed DQ8/Ins complexes in R3

DQ8-Ins	Peptide*	T1D#10 C8 [†]	T1D#3 C8	T1D#4 C5
I72C-R1	<u>LE</u> EALYLVA <u>EE</u> C	—	—	—
I72C-R2	VEALYLVA <u>GE</u> RC	—	—	—
I72C-R3	VE <u>E</u> LYLVAGE <u>EG</u> C	1.5	31.6	4.9
I72C-R4	VE <u>A</u> EYLVA <u>GE</u> RE <u>G</u> C	—	0.4	0.1
V65C-R1	<u>LE</u> EALYL <u>CV</u> A <u>EE</u> R	—	—	—
V65C-R2	VEALYL <u>C</u> AG <u>ER</u> G	—	—	—
V65C-R3	VE <u>E</u> LYLV <u>CG</u> EE <u>GG</u>	2.3	38.8	0.4
V65C-R4	VE <u>A</u> EYLVA <u>C</u> ER <u>EG</u> G	—	—	—

The lowest concentration of IFN- γ standard was 0.017 ng/mL and readings below this level are indicated as with an "—."

*To "trap" each peptide in a specific register, corresponding p1 and p9 anchor residues of the peptide were mutated to E (in bold). To "fix" the peptide in a specific register by disulfide bonding, each set of peptides had a single C residue (underlined) at position p6 (to bind to V65C) or at p11 (to bind to I72C). The other C residue that would not be at p6 was changed to A (italic).

[†]T-cell clones were stimulated with B6K10 cells expressing the indicated register-fixed constructs and assayed for secreted human IFN- γ (ng/mL).

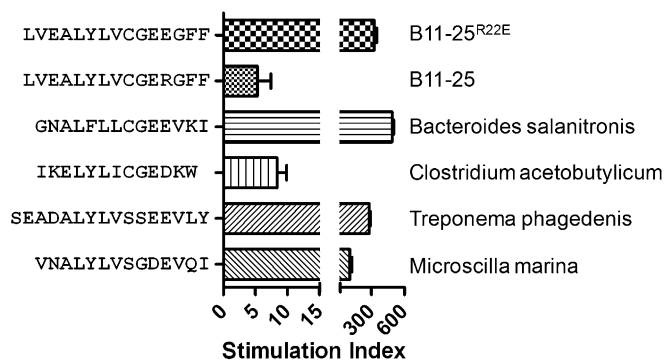


Fig. 4. Stimulation index for a representative T-cell clone activated *in vitro* using 3 μ M of B:11-23^{R22E} peptide, B:11-23 peptide, or homologous microbial peptides, respectively. Data are mean \pm SD of duplicates.

of fairly low mean fluorescence intensity) and only detectable in 6 of 16 T1D subjects. Interestingly, five of the six subjects who responded to B:11-23 had the susceptible *VNTR* genotype, suggesting that levels of thymic insulin expression as dictated by the insulin *VNTR* polymorphism may influence the selection of B:11-23-specific T cells (27). In earlier experiments with the DR0401/preproinsulin tetramers, an average of 14% tetramer-positive T cells with a positive response rate of 75% in T1D subjects was observed under similar experimental conditions (28). This comparison of DR- and DQ-restricted insulin responses further underscores the apparent rarity of these DQ8 T cells in the periphery, although it could be argued that our tetramer reagents are unable to detect a subset of T cells that recognize this epitope with lower functional avidity. It is plausible that these T cells may exist at higher frequencies close to disease onset but are subsequently lost in patients because of insufficient levels of antigen to maintain their peripheral survival as a consequence of β -cell destruction (29–31). It is also possible that B:11-23-specific T cells that remain in subjects with long-standing disease preferentially reside in islets or secondary lymphoid tissue, and are therefore virtually undetectable in peripheral blood. Additional studies in at-risk subjects would be necessary to differentiate between these possibilities.

Although our T-cell clones responded to the denatured insulin protein and wild-type insulin peptide, none recognized any peptides from naturally processed native insulin. DeINS was generated in the presence of β -mercaptoethanol, which would be expected to reduce disulfide bonds and produce the free insulin A and B chains. Lack of reactivity to processed native insulin suggests that the disulfide bonds within the native structure of insulin protein may limit effective generation of the B:11-23 epitope. This behavior would be consistent with that of the “type B” T-cell clones described by Mohan et al., which respond only to peptides offered exogenously to the APC but not to naturally processed protein (32, 33). In any case, the binding register of B:11-23 that was recognized by our DQ8-restricted clones was not the high-affinity binding register. As more fully detailed in *Results*, the profound influence of substitutions between positions 14 and 22 (most notably residues 16, 18, and 21 for the B:11-23^{R22E} peptide and residues 14 and 22 for the B:11-23 peptide) strongly implicate A14, L17, C19, and R22 as the immunogenic anchor residues and L15, Y16, V18, and E21 as important TCR contacts. Thus, the DQ8-restricted T cells we isolated appear to recognize B:11-23 presented in the suboptimal R3 register. This contention was further confirmed by experiments in which the insulin B:11-23 peptide was fixed in the R3 register by disulfide cross-linking between the linked peptide and DQ8 molecule. Thus, in both mice and humans, autoreactive T cells recognize the B:11-23 peptide presented in R3 (9, 10).

A long-standing puzzle in autoimmunity has been how the T cells that drive these diseases escape negative selection in the thymus and peripheral regulatory mechanisms to find an activating antigen in their target organs. It is likely that the interplay among genetic, epigenetic, and environmental factors ultimately play a role (34, 35). However, in most cases the mechanism does not seem to be via a general breakdown in thymic deletion or peripheral regulatory mechanisms, but rather is related to MHC allele-specific antigen presentation. In this regard, there have been several mechanisms suggested, including differential gene expression in the thymus versus periphery (36, 37), posttranslational modifications (38–40), and activation by molecular mimicry [e.g., high-affinity cross-reactive peptides from inflammatory microorganisms (41)]. Without excluding other mechanisms, our data raise the possibility that this third mechanism might be at work in T1D. We identified a number of environmental microbes expressing proteins that contain peptide sequences with high homology to the R3 core region of B:11-23^{R22E} peptide (ALYLVCGER). These microbes included species that are commonly isolated from poultry or dairy animals and environmental microbes that are commonly encountered (23–26). DQ8-restricted insulin-specific T-cell clones were activated by these homologous microbial peptides as effectively as the B:11-23^{R22E} peptide. Thus, it is possible that responses to epitopes from these or other similar microbes could play a role in initiating the activation and expansion of insulin reactive T cells.

In total, our study verifies the importance of T-cell responses to antigenic peptides with low-affinity HLA binding in human autoimmune disease, documenting the presence of DQ8-restricted insulin B:11-23-specific T cells in subjects with T1D by directly visualizing these cells with DQ8/B:11-23^{R22E} tetramers. These T cells were only detected in patients and also responded to stimulation with homologous peptides derived from commonly encountered microbial antigens. These observations highlight the possibility that self-reactive T cells could be activated by molecular mimicry early in the autoimmune process, leading to a subsequent breakdown in self-tolerance. Our results are in agreement with observations for the IA^{g7}-restricted T cells of NOD mice (9, 10), highlighting a mechanism shared by mouse and man through which T cells that recognize a weakly bound peptide can circumvent tolerance mechanisms and play a role in the initiation of autoimmune diseases, such as T1D. Given that B:11-23-specific T cells can be successfully visualized using tetramers, tetramer-based approaches could be useful to monitor responses to this peptide in prediabetic subjects, over the course of immune-modulatory therapy, or following the administration of tolerogenic vaccines (42, 43).

Materials and Methods

Human Subjects. Fresh blood samples were obtained from healthy individuals and subjects with T1D who had HLA-DQA1*03:01/DQB1*03:02 (DQ8) haplotypes as part of a Benaroya Research Institutional Review Board-approved study (R00123A) after obtaining written consent. The *VNTR* genotype was assigned based on the genotype at the –23 A/T single nucleotide polymorphism of the *INS* promoter (44). The attributes of the subjects recruited for the study are summarized in Table S1. Subjects with T1D were sampled within 4 y of diagnosis. Two DQ8 subjects with type 2 diabetes that received regular insulin injections to maintain glycemic control were also recruited.

Antibodies and Tetramer Reagents. The following fluorescent antibodies were used: anti-human CD3-FITC and CD25-APC (eBioscience), CD4-PerCP (BD Biosciences), anti-HLA-DQ-PE (Leinco Technologies), and streptavidin-R-PE (Life Technology). HLA-DR and HLA-DQ blocking antibodies (L243 and SPV13) were produced from hybridomas obtained from the ATCC. The B7/21 anti-DP producing hybridoma was a gift from Alessandro Sette (La Jolla Institute for Allergy and Immunology, La Jolla, CA). These antibodies were purified using protein A-Sepharose beads. Tetramers for staining T cells were generated as previously described (45, 46).

Peptides and deINS Protein. Insulin B: 11-23 (B:11-23), LVEALYLVCGERG, its modified version, Insulin B:11-23, R22E (B:11-23^{R22E}), LVEALYLVCGERG,

libraries of substituted peptides, and peptides derived from bacterial antigens (detailed in Figs. 3 and 4) were purchased from Mimotopes. Peptides were dissolved in DMSO at 20 mg/mL (~12 mM). Insulin powder was purchased from Sigma-Aldrich. DeINS protein was prepared by dissolving 50 mg of insulin powder into 500 μ L of 8 M Urea plus 10 μ L of β -mercaptoethanol followed by dialysis into PBS in 0.5 mL capacity of Slide-A-Lyzer Dialysis Cassettes, 3K MWCO (Thermo Scientific). After dialysis, the denatured protein was collected and stored as aliquots at -20°C .

Register-Fixed Constructs: Generation, Expression, and T-Cell Stimulation. Murine stem cell virus-based retroviral expression vectors encoding the DQ8/Ins complexes were constructed using standard molecular biology procedures. The constructs encode the complexes as a single ORF, comprising the DQ8 α - and β -chains separated by a picornavirus 2A peptide (47), and an internal ribosome entry site-driven GFP. The insulin peptide is fused via a flexible linker to the N terminus of the mature β -chain (48) (Fig. S2A). B6K10 cells were transduced

with the resulting recombinant retroviruses and used without further enrichment.

T-cell stimulation assays were performed by coculturing 10^5 T cells and 10^5 transduced B6K10 cells in 200 μ L of medium for 16–24 h. Assays were performed in triplicate and 100 μ L of the supernatant was assayed using the human IFN- γ ELISA Ready-SET-Go kit (eBioscience), according to the manufacturer's recommendations.

Methods for peptide binding measurements, CD4 T in vitro stimulation, tetramer staining, insulin-specific CD4⁺ T-cell cloning, and T-cell proliferation assays are available in *SI Materials and Methods*.

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